IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Denise L. Faustman
Serial No.:	09/913,664
Filed:	August 17, 2001
Entitled:	METHOD FOR INHIBITING TRANSPLANT REJECTION

ART UNIT: 1651

EXAMINER: V. Afremova

Atty. Docket No.: DLF-002.1P US

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

DECLARATION OF DENISE L. FAUSTMAN UNDER 37 C.F.R. §1.132

I, Denise L. Faustman, hereby declare and state that:

1. I am the inventor and owner of the subject matter claimed in the above-identified patent application, U.S. Serial No. 09/913,664.

2. I am an Associate Professor of Medicine at Harvard Medical School and Director of the Immunobiology Laboratory at the Massachusetts General Hospital (MGH), Charlestown, MA.

3. The present application is directed to an improved method for the transplantation of allogeneic or xenogeneic donor tissue into a host recipient. Specifically, a method is disclosed whereby viable (live) donor tissue is treated with at least one enzyme to temporarily remove surface antigens, particularly MHC Class I surface antigens, prior to the transplantation step without rendering the donor tissue non-viable. The removal of the surface antigens helps attenuate the immune response of the host and facilitates acceptance of the foreign donor tissue. Once the viable transplant tissue is established in the host, MHC Class I surface antigens are reexpressed on the surface of the cells of the

donor tissue and are recognized by the host organism as "self" antigens, thereby preventing attack of the donor tissue by the immune system of the host. In a preferred method, the donor tissue is treated with either papain alone or a combination of papain and _-galactosidase.

4. I have been informed by my attorney, and it is my understanding, that the claims of my application have been rejected as lacking novelty in view of the teachings of two U.S. patents, namely, U.S. 4,399,123 to Oliver et al. (the '123 patent) and U.S. 5,397,353 also to Oliver et al. (the '353 patent), or as being obvious in view of the teachings of those Oliver et al. patents taken together with other references, namely, Galati et al. (Cytometry, 27:77-83, 1997) and Stone et al. (Transplantation, 65(12):1577-83, June 1998). I am familiar with these publications, having studied them in connection helping my attorneys answer an earlier office action issued in my application.

5. These references do not teach my invention or make it obvious because the two Oliver et al. patents (the '123 patent and the '353 patent) and also the Stone et al. article teach a treatment for donor tissue intended for transpant that kills all living cells associated with that tissue (and thus the donor tissue is not viable at the time of transplant). Furthermore, the Galati et al. article does not relate to preparation of cells for transplantation but rather to a method for obtaining MHC Class I antigen complexes removed from cells by papain digestion, as a means of quantitatively measuring the amount of MHC Class I molecules expressed.

6. I have been informed and believe that the Examiner has maintained the rejections based on the teachings of these documents, and I have been informed and believe that the Examiner has argued that the treatments of donor tissue taught in the '123 patent, the '353 patent, and the Stone et al. article are not disclosed to be lethal to donor tissues.

7. I am making this declaration to demonstrate that the treatments of donor tissues as described in the '123 patent, the '353 patent, and the Stone et al. article directly cause the tissue to quickly become non-viable and therefore unsuitable for use in my invention.

8. I personally conducted experiments following the teachings of the '123 patent, the '353 patent, and the Stone et al. article to treat murine and human eukaryotic cells with varying concentrations of the agents taught in those references. Specifically, I treated murine T cells isolated from splenocytes, kidney cells, and liver cells, or human peripheral blood lymphocytes (PBLs) with varying concentrations of sodium azide (as taught in both the '123 patent and the '353 patent), or with formaldehyde (taught in the '123 patent), or with acetone (taught in the '353 patent), or with alcohol + α-galactosidase (as taught in the Stone et al. document). The data are presented herein.

9. **I. Treatment of donor cells with sodium azide**

Example 1 of the '123 patent specifies that tissue intended for transplantation (human dermis) is treated with trypsin (2 mg/ml in 0.1M phosphate buffer) and 0.5 mg/ml sodium azide at 15° C for 28 days, prior to glutaraldehyde treatment (16 hours) and implantation into a host. Example 2 of the '123 patent specifies the same treatment, substituting chymotrypsin for the trypsin. Example 3 of the '123 patent specifies the same treatment, substituting rat tendon tissue for the human dermis of Example 1. Example 4 of the '123 patent specifies the same treatment using pig ligament in place of human dermis tissue. Example 6 of the '123 patent specifies the same treatment using pig dermis.

Example 1 of the '353 patent specifies treatment of pig dermis intended for transplant by immersion in acetone (1 hour), followed by washing in 0.1M phosphate buffer, then treatment with trypsin (2 mg/ml in 0.1M phosphate buffer) and 0.5 mg/ml sodium azide at 15° C for 28 days. Example 2 of the '353 patent specifies treatment of pig dermis intended for transplant by immersion in acetone for 2 hours, followed by washing in 0.1M phosphate buffer, then treatment with papain (3 mg/ml in 0.1M phosphate buffer), cysteine (0.01M), and 0.5 mg/ml sodium azide for 28 days. Example 3 of the '353 patent specifies treatment of pig dermis intended for transplant by immersion in acetone (39 hours), followed by washing in 0.1M phosphate buffer containing sodium azide (0.5 mg/ml) and trypsin (50 g/l) for 7 days, then dehaired and treated with the trypsin/sodium azide buffer for an additional 21 days.

Thus, the '123 and '353 patents both teach a protocol for treating donor tissue prior to transplant that requires contact of the tissue with sodium azide at a concentration of 0.5 mg/ml for about 28 days.

The '123 and '353 patents state that the sodium azide is included as a bactericide, however I followed the '123 and '353 patent teachings in order to demonstrate that the sodium azide treatment also results in the production of non-viable donor tissue for the transplant:

Protocol: Fresh murine T cells isolated from NOD and B6 mouse splenocytes, kidney cells and liver cells of B6 mice, and human peripheral blood lymphocytes (PBLs) were isolated and plated into 24-well plates at a density of 1×10^6 cells per ml.

Cells were incubated with sodium azide (NaN_3) at a particular concentration at 15° C. NaN_3 concentrations of 0 mg/ml, 0.001 mg/ml, 0.002 mg/ml, 0.01 mg/ml, 0.02 mg/ml, 0.1 mg/ml, and 0.5 mg/ml were tested. The cells were suspended in standard tissue culture media (RPMI with 10% FCS). The % viability of the cells as a function of sodium azide concentration was assessed after 22 hours and after 3 days incubation by flow cytometry using propidium iodide. Results of these experiments are shown in Tables 1A, 1B, and 1C.

Results: Table 1A shows the % viability of murine NOD and B6 T cells isolated from splenocytes and treated with varying concentrations of sodium azide. Table 1B shows the % viability of murine B6 T cells isolated from kidney and liver cells and treated with varying concentrations of sodium azide. Table 1C shows the % viability of human peripheral blood lymphocytes treated with varying concentrations of sodium azide.

Table 1A:
Sodium azide toxicity on T cells isolated from splenocytes of NOD and B6 mice

NaN_3 Concentration (mg/ml)	% viable cells (NOD/B6) at 22 hours	% viable cells (NOD/B6) at 3 days
0	75/80	65/72
0.001	55/65	0/0
0.002	50/61	0/0
0.01	50/55	0/0
0.02	21/35	0/0
0.10	5/5	0/0
0.50	4/2	1/0

Table 1B:

Sodium azide toxicity on liver and kidney cells from B6 mice

NaN_3 Concentration (mg/ml)	% viable cells (liver/kidney) at 22 hours	% viable cells (liver/kidney) at 3 days
0	85/82	81/82
0.001	20/14	2/5
0.002	20/15	1/0
0.01	5/4	0/0
0.02	1/2	0/0
0.10	0/0	0/0
0.50	0/0	0/0

Table 1C:

Sodium azide toxicity on human PBLs

NaN_3 Concentration (mg/ml)	% viable PBLs at 22 hours	% viable PBLs at 3 days
0	92	87
0.001	12	2
0.002	8	2
0.01	7	2
0.02	7	0
0.10	5	0
0.50	0	0

10. My experiments show that sodium azide treatment was very toxic to both freshly isolated murine and human cells at all the test concentrations of 0.001–0.5 mg/ml. After 3 days of exposure to sodium azide, all donor cells exposed to sodium azide concentrations above 0.01 mg/ml were uniformly and completely non-viable. All sodium azide experiments were terminated after 3 days as less than 5% viable cells remained in the tissue culture wells at all sodium azide concentrations (except control cells: 0 mg/ml sodium azide).

11. **II. Treatment of donor cells with formaldehyde**

The '123 patent teaches a method for preparing fibrous tissue for transplantation which includes two enzymatic treatments (proteolytic and carbohydrate-splitting enzymes), followed by contact with a crosslinking agent, i.e., glutaraldehyde or formaldehyde at a concentration greater than 0.01% (see, col. 3 (lines 45-65); col. 4 (lines 49-65) of the '123 patent). The '123 patent further specifies that treated tissue is only suitable for transplant "after its sterilization" (see, col. 3 (line 43 and line 48); col. 4 (line 53); col. 5

(line 2 and lines 26-30) of the '123 patent). Example 5 of the '123 patent specifies that samples of pig dermal collagen were treated with 0.1%, 1% or 5% formaldehyde for 21 days.

The '123 patent states that the formaldehyde treatment is to remove antigenicity of the treated tissue by crosslinking amino groups in the tissue (see, col. 3 (line 60) to col. 4 (line 7)); however, I followed the '123 patent teachings with respect to formaldehyde treatment in order to demonstrate that exposure to such crosslinking agents as glutaraldehyde or formaldehyde also results in the production of non-viable donor tissue for the transplant:

Protocol: Fresh murine T cells isolated from NOD and B6 mouse splenocytes, kidney cells and liver cells of B6 mice, and human peripheral blood lymphocytes (PBLs) were isolated and plated into 24-well plates at a density of 1×10^6 cells per ml.

The cells were treated for 5 minutes in either 0.1%, 0.2%, or 5% formaldehyde. The % viability of the cells as a function of formaldehyde concentration was assessed after 22 hours and after 3 days by flow cytometry using propidium iodide. Results of these experiments are shown in Tables 2A, 2B, and 2C.

Results: Table 2A shows the % viability of T cells isolated from murine NOD and B6 splenocytes 22 hours and 3 days after a 5 minute treatment with formaldehyde. Table 2B shows the % viability of murine B6 kidney and liver cells 22 hours and 3 days after a 5 minute treatment with formaldehyde. Table 2C shows the % viability of human peripheral blood lymphocytes 22 hours and 3 days after a 5 minute treatment with formaldehyde

Table 2A:

Formaldehyde toxicity in T cells isolated from splenocytes of NOD and B6 mice

Formaldehyde Concentration (%)	% viable cells (NOD/B6) at 22 hours	% viable cells (NOD/B6) at 3 days
0	78/86	70/78
0.1	11/15	0/0
0.2	8/14	0/0
5	0/0	0/0

Table 2B:

Formaldehyde toxicity in T cells isolated from liver and kidney cells of B6 mice

Formaldehyde Concentration (%)	% viable cells (liver/kidney) at 22 hours	% viable cells (liver/kidney) at 3 days
0	91/95	88/90
0.1	2/3	0/0
0.2	0	0/0
5	0	0/0

Table 2C:

Formaldehyde toxicity in fresh human PBLs

Formaldehyde Concentration (%)	% viable PBLs at 22 hours	% viable PBLs at 3 days
0	95	90
0.1	5	0
0.2	4	0
5	0	0

12. My experiments show that donor cells treated for only five minutes (as compared with 16 hours glutaraldehyde treatment (Examples 1-4 and 6) or 21 days formaldehyde treatment (Example 5) in the '123 patent) results in completely non-viable tissue in all cases within 3 days at concentrations of 0.1% or greater.

13. **III. Treatment of donor cells with acetone**

The '353 patent teaches a wet preparation of tissue intended for transplant involving initial extraction of the tissue with an organic solvent such as acetone (see, col. 4 (line 50) of the '353 patent) before sterilization with gamma radiation or hydrogen peroxide. Example 1 of the '353 patent specifies treatment of pig dermis intended for transplant by immersion in acetone for 1 hour, followed by washing and enzyme treatment. Example 2 of the '353 patent specifies treatment of pig dermis intended for transplant by immersion in acetone for 2 hours, followed by washing and enzyme treatment. Example 3 of the '353 patent specifies treatment of pig dermis intended for transplant by immersion in acetone for 39 hours, followed by washing and enzyme treatment.

The '353 patent thus teaches the use of acetone in every one of its examples of tissue preparation. I followed the '353 patent teachings with respect to acetone treatment in

order to demonstrate that exposure to such agents as acetone also results in the production of non-viable donor tissue for the transplant:

Protocol: Fresh murine T cells isolated from NOD and B6 mouse splenocytes, kidney cells and liver cells of B6 mice, and human peripheral blood lymphocytes (PBLs) were isolated and plated into 24-well plates at a density of 1×10^6 cells per ml. All cells were incubated with acetone for 1 hour and rinsed once in RPMI. The % viability of the cells as a function of the presence or absence of acetone was assessed after 22 hours by flow cytometry using propidium iodide. Results of these experiments are shown in Tables 3A, 3B, and 3C.

Results: Table 3A shows the % viability of T cells isolated from murine NOD and B6 splenocytes 22 hours after incubation for 1 hour with or without acetone. Table 3B shows % viability of murine B6 kidney and liver cells 22 hours after incubation for 1 hour with or without acetone. Table 3C shows % viability of human peripheral blood lymphocytes 22 hours after incubation for 1 hour with or without acetone.

Table 3A:

Acetone toxicity (1 hr) in T cells isolated from splenocytes of NOD and B6 mice

1-hour Acetone Exposure	% viable cells (NOD/B6) after 22 hours
no (control)	72/81
yes	0/0

Table 3B:

Acetone toxicity (1 hr) in liver and kidney cells of B6 mice

1-hour Acetone Exposure	% viable cells (liver/kidney) after 22 hours
no (control)	79/89
yes	0/0

Table 3C:

Acetone exposure (1 hr) on fresh human PBLs

1-hour Acetone Exposure	% viable cells (PBLs) after 22 hours
no (control)	93
yes	0

14. My experiments show that donor cells treated for one hour (cf. Example 1 of the '353 patent) results in completely non-viable tissue in all cases within 22 hours after acetone extraction.

15. The foregoing experiments show that following the teachings of the two patents of Oliver et al. (the '123 and the '353 patent) with respect to preparation of tissue for transplant results in non-viable tissue, which is not suitable for use in accordance with my invention.

16. **IV. Treatment of donor cells with alcohol + α-galactosidase**

The Stone et al. article teaches a method for preparing porcine articular cartilage for transplant. Cartilage plugs were immersed in alcohol for 5 minutes, then immersed in a phosphate-citrate-sodium chloride buffer containing 100 U/ml of α-galactosidase and incubated for 4 hours at 26° C. (See, Stone et al., page 1578, right column.)

The Stone et al. article states that the alcohol treatment of the donor tissue is to remove synovial fluid and lipid-soluble contaminants (page 1578, right column); however, I followed the Stone et al. teachings with respect to alcohol treatment in order to demonstrate that exposure to such reagents also results in the production of non-viable donor tissue for the transplant:

Protocol: Fresh murine T cells isolated from NOD and B6 mouse splenocytes, kidney cells and liver cells of B6 mice, and human peripheral blood lymphocytes (PBLs) were isolated and plated into 24-well plates at a density of 1×10^6 cells per ml. As in the Stone et al. article, the cells were exposed to alcohol for 5 minutes, washed and then incubated with 100 U/ml of α-galactosidase in phosphate-citrate-sodium chloride buffer for 4 hours at 26° C. Comparative wells of cells were also incubated in alcohol alone (followed by buffer only wash) or in the α-galactosidase buffer alone. The % viability was determined using flow cytometry after the 4-hour incubation. Results of these experiments are shown in Tables 4A, 4B, and 4C.

Results: Table 4A shows the % viability of T cells isolated from murine NOD and B6 splenocytes after 4 hours of incubation at 26° C with either alcohol alone, α-

galactosidase alone, or a combination of alcohol + α-galactosidase. Table 4B shows the % viability of murine B6 kidney and liver cells after 4 hours incubation at 26° C with either alcohol alone, α-galactosidase alone, or a combination of alcohol + α-galactosidase. Table 4C shows the % viability of human peripheral blood lymphocytes after incubation for 4 hours at 26° C with alcohol alone, α-galactosidase alone, or a combination of alcohol + α-galactosidase.

Table 4A:

Alcohol + α-galactosidase toxicity in T cells isolated from NOD and B6 splenocytes

Treatment	% viable cells (NOD/B6) after 4 hours
buffer only	92/94
alcohol alone (then buffer)	0/0
α-galactosidase buffer alone	89/91
alcohol + α-galactosidase	0/0

Table 4B:

Alcohol + α-galactosidase toxicity in B6 liver and kidney cells

Treatment	% viable cells (liver/kidney) after 4 hours
buffer only	98/91
alcohol alone (then buffer)	0/0
α-galactosidase buffer alone	95/90
alcohol + α-galactosidase	0/0

Table 4C:

Alcohol + α-galactosidase toxicity in human PBLs

Treatment	% viable cells (NOD/B6) after 4 hours
buffer only	98
alcohol alone (then buffer)	0
α-galactosidase buffer alone	95
alcohol + α-galactosidase	0

17. My experiments show that treatment of donor tissue for only five minutes with alcohol (as described in Stone et al.) results in non-viable tissue in all cases within four hours after alcohol treatment. Exposure of tissues to α-galactosidase alone does not appear to be toxic to donor tissues.

18. The foregoing experiments show that following the teachings of the Stone et al. article with respect to preparation of tissue for transplant results in non-viable tissue, which is not suitable for use in accordance with my invention.

19. Conclusions

(a) As seen in Tables 1A-1C, treatment of viable tissues with sodium azide at concentrations as low as 0.001mg/ml (that is, 500 times less concentrated than taught by Oliver et al.) results in the production of 100% non-viable tissue. The absence of any viable cells was observed as early as 22 hours post-treatment, and all treated cells were non-viable by 3 days post-treatment (that is, within a period 24 days shorter than taught by Oliver et al.).

(b) As seen in Tables 2A-2C, treatment of viable tissues with an aldehyde cross-linking agent such as formaldehyde for as little as 5 minutes (as compared with incubation for hours or weeks taught by Oliver et al.) results in the production of 100% non-viable tissue after incubation in concentrations as low as 0.1%. The absence of any viable cells was observed as early as 22 hours post-treatment, and all treated cells were non-viable by 3 days post-treatment.

(c) As seen in Tables 3A-3C, treatment of viable tissue with acetone for 1 hour (as taught in the '353 patent) results in 100% non-viable tissue within 22 hours post-treatment.

(d) As seen in Tables 4A-4B, treatment of viable tissues with alcohol for as little as 5 minutes results in 100% non-viable tissue within 4 hours post-treatment.

20. The foregoing experiments confirm that the teachings of the '123 patent, the '353 patent, and the Stone et al. article teach treatments for intended transplant tissue which render the tissue non-viable almost immediately. In contrast to this, my invention requires that the pre-tranplant treatment of viable donor tissue leaves the tissue viable, and that the viability is maintained after transplant. Thus, the Oliver et al. patents do not teach my invention. Moreover, combining the teachings of the Oliver et al. patents with the Galati et al. and the Stone et al. articles still results in the teaching of a pre-treatment for donor tissue that leads to non-viable tissue prior to transplant, and therefore that combination of publications cannot render my invention obvious.

21. I further declare that all statements made herein of my own knowledge are true and that statements made on information and belief are believed to be true and further that false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

3/16/04
date

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